34. F. P. Roth, J. D. Hughes, P. W. Estep, G. M. Church, *Nature Biotechnol.* **16**, 939 (1998).
35. In Fig. 2, deletion strains grown in −gal are displayed relative to *wt*−gal conditions by subtracting the $\log_{10}$ expression ratio of *wt*−gal versus reference from the $\log_{10}$ expression ratio of the deletion strain versus reference. As predicted and confirmed experimentally (T. Ideker, V. Thorsson, data not shown), this operation results in approximately a $\sqrt{2}$-fold increase in error.
36. M. Ashburner *et al.*, *Nature Genet.* **25**, 25 (2000). [www.geneontology.org/]
37. K. Mehlhorn, S. Naeher, *The LEDA Platform of Combinatorial and Geometric Computing* (Cambridge Univ. Press, Cambridge, 1999). [www.algorithmic-solutions.com/]
38. J. Felsenstein, *Cladistics* **5**, 164 (1989).
39. M. B. Eisen, P. T. Spellman, P. O. Brown, D. Botstein, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14863 (1998).
40. We thank G. van den Engh for ideas on visual display; B. Schwikowski and P. Uetz for early access to protein interactions; A. Siegel, K. Y. Yeung, E. Cho, Q. Zong, and E. Yi for assistance with global analysis; T. Young, D. Morris, K. Dimitrov, M. Johnston, and I. Sadowski for informative discussions; and J. Hopper, J. Aitchison, and L. Kruglyak for critical reading of the manuscript. This work was funded by grants from NIH (T.I., J.A.R., R.A.) and the Sloan Foundation/U.S. Department of Energy (V.T.).

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Vital Involvement of a Natural Killer Cell Activation Receptor in Resistance to Viral Infection

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Natural killer (NK) cells are lymphocytes that can be distinguished from T and B cells through their involvement in innate immunity and their lack of rearranged antigen receptors. Although NK cells and their receptors were initially characterized in terms of tumor killing in vitro, we have determined that the NK cell activation receptor, Ly-49H, is critically involved in resistance to murine cytomegalovirus in vivo. Ly-49H requires an immunoreceptor tyrosine-based activation motif (ITAM)–containing transmembrane molecule for expression and signal transduction. Thus, NK cells use receptors functionally resembling ITAM-coupled T and B cell antigen receptors to provide vital innate host defense.

Natural killer (NK) cells were first identified because of their "natural" ability to kill tumors in vitro, an ability that is now known to occur through activation receptors that trigger the release of perforin-containing cytolytic granules [reviewed in (*1*)]. These lymphocytes can be distinguished from T and B cells because they do not express rearranged antigen receptors and are not directly involved in acquired immunity. However, NK cells participate in early innate host defense against pathogens and are generally thought to counter infections through a nonspecific response to inflammatory cytokines that induce their production of interferon-γ (*2*). Yet, NK cells appear to respond specifically against certain pathogens. For example, in humans, selective NK cell deficiency is associated with recurrent systemic infections, especially with herpesviruses such as cytomegalovirus (*3*). This is closely paralleled by the susceptibility of NK cell–depleted mice to murine cytomegalovirus (MCMV) but not to lymphocytic choriomeningitis virus (*4*). Although the mechanisms underlying this susceptibility are incompletely understood, NK cell receptors that activate tumor cytotoxicity may play important roles in innate defense against specific infections (*5*).

The critical involvement of NK cell activation receptors in defense against pathogens is highlighted by the expression of virus-encoded proteins that interfere with natural killing (*6*). In many cases, these proteins enhance the function of inhibitory major histocompatibility complex (MHC) class I–specific NK cell receptors that potentially interfere with signals from activation receptors, such as Ly-49D and Ly-49H, that are coupled to immunoreceptor tyrosine-based activation motif (ITAM)–containing transmembrane

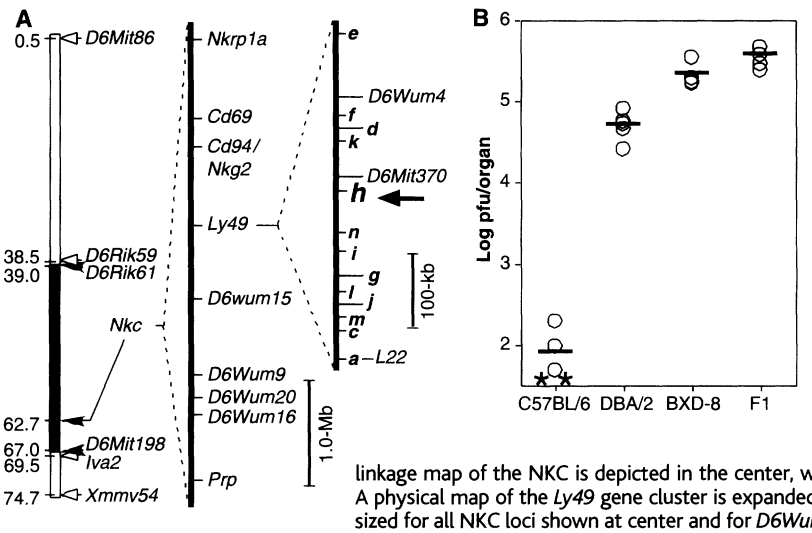

Fig 1. BXD-8 mice possess the NKC^{B6} haplotype and display MCMV susceptibility that is not complemented by DBA/2. (**A**) Genetic and physical maps of NKC-linked loci on mouse chromosome 6. A genetic linkage map and schematic diagram of the BXD-8 chimeric chromosome 6 is represented at left, based on the Mouse Genome Informatics database (*14*, *25*). Chromosomal regions derived from the C57BL/6 (solid bar) or DBA/2 (open bars) inbred progenitor strains are indicated, along with genetic position (distance is indicated in centimorgans from the centromere) of C57BL/6 alleles (solid arrowheads) and DBA/2 (open arrowheads) alleles for the recombination breakpoints. Sequence-tagged site markers that reside close to the centromere and telomere are also shown. Thus far, in BXD-8, all loci reported to reside between *D6Mit86* and *D6Rik59* (36 tested loci) and between *Iva2* and *Xmmv54* (10 loci) contain DBA/2 alleles. C57BL/6 alleles account for all BXD-8 loci reported to reside between *D6Rik61* and *D6Mit198*, except for the *Cmv1* locus (*9*, *25*). A physical linkage map of the NKC is depicted in the center, with selected loci that have been useful to distinguish alleles (*19*). A physical map of the *Ly49* gene cluster is expanded at right (*26*, *27*). BXD-8 and C57BL/6 NKC alleles are identically sized for all NKC loci shown at center and for *D6Wum4*, *D6Mit370*, *Ly49g*, and *Ly49a* (*L22*). Surrounding *Ly49h* are the *Ly49k* and *Ly49n* pseudogenes (*28*). (**B**) MCMV replication in F_1 hybrid offspring from DBA/2 and BXD-8. Three days after infection [with MCMV Smith strain, 2×10^4 plaque-forming units (PFUs)], organ viral titers were assessed in tissue homogenates collected from C57BL/6, DBA/2, BXD-8, and (DBA/2 × BXD-8) F_1 hybrid mice (five mice per group), as indicated. Spleen titers are shown here; liver titers are available online (*8*). Each point represents the average titer determined for an individual mouse. In the spleens of two C57BL/6-infected mice, viral replication was below the level of detection by this assay and is indicated with asterisks. Mean viral titers for each group are depicted as horizontal bars. For mice with titers below the level of detection of the assay, the minimum number of detectable PFUs (10^2) was assumed to determine the mean. This assumption overestimates the mean for the group having titers below detectable levels.

molecules (*1*). To date, however, the in vivo role of these NK cell activation receptors has not been determined.

Certain strains of mice are resistant to MCMV infection in vivo, whereas others are susceptible (*7*, *8*). Resistant strains become susceptible when NK cells are depleted (*9*), succumbing to infection and manifesting high viral titers in the spleen, a phenotype also seen with perforin deficiency (*5*). Extensive genetic mapping studies have indicated that a single autosomal dominant locus, termed *Cmv1*, controls both survival and viral titers in the spleen but not the liver (*7*, *10–12*). Because *Cmv1* maps to the NK gene complex (NKC) on mouse chromosome 6 and the NKC contains clusters of highly related genes encoding NK cell receptors, including the Ly-49 family (*13*), we hypothesized that a polymorphic NK cell receptor gene may account for resistance to MCMV.

Further dissection of *Cmv1* was complicated by the large size of the NKC, with its high density of NK cell receptor genes, already known allelic polymorphisms, and recombination hotspots (*13*), thwarting direct sequence comparison or more extensive genetic mapping approaches. However, the BXD-8 recombinant inbred mouse, derived from resistant C57BL/6 (*Cmv1*^{r}) and susceptible DBA/2 (*Cmv1*^{s}) strains, appeared to have inherited the entire NKC and flanking genomic segments from C57BL/6 (NKC^{B6} haplotype) (Fig. 1A) (*14*, *25*). Yet, these mice displayed the susceptible phenotype (*9*). (DBA/2 × BXD-8)F_1 hybrid mice also displayed high viral titers in the spleen (Fig. 1B), demonstrating the absence of complementation by DBA/2 and suggesting that BXD-8 mice may selectively lack expression of the relevant C57BL/6-derived NKC gene for MCMV resistance.

In candidate NKC gene expression analyses, we determined that NK cells from BXD-8 mice lacked reactivity with monoclonal antibody (mAb) 3D10, which is specific for Ly-49H (Fig. 2A) (*15*). Yet, BXD-8 NK cells express allotypic epitopes for all other serologically detectable C57BL/6-derived Ly-49 molecules, including Ly-49D, whose gene is within ~50 kb of *Ly49h*, suggesting that BXD-8 mice are selectively deficient in Ly-49H expression (Fig. 2A) (*8*). Furthermore, the mAb to Ly-49H (anti–Ly-49H) does not stimulate redirected lysis of human Daudi targets by BXD-8 NK cells, although other in vitro functions remain intact (Fig. 2B) (*8*). The absence of anti–Ly-49H reactivity was not due to the lack of an allotypic epitope on an expressed molecule, because

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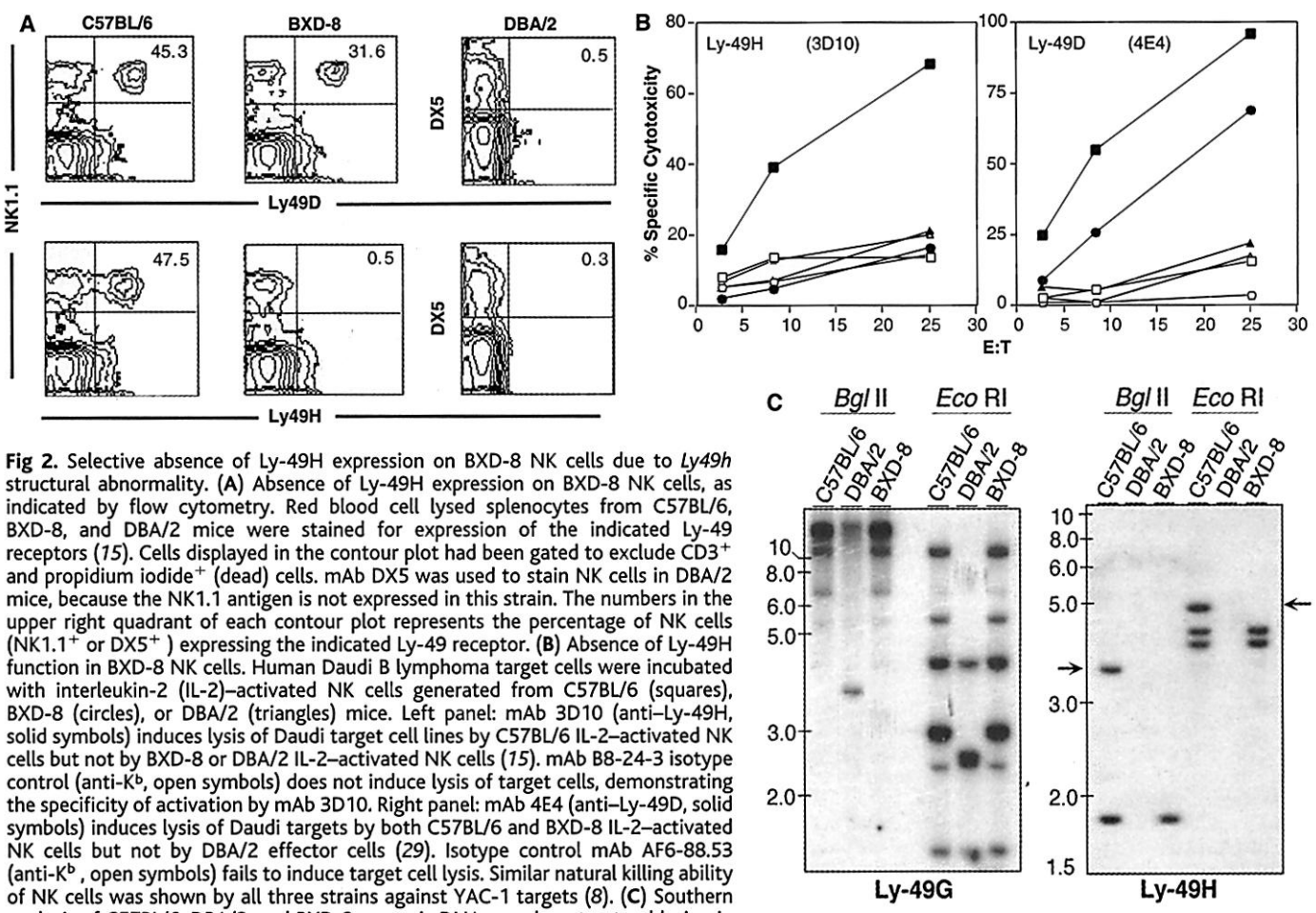

Fig 2. Selective absence of Ly-49H expression on BXD-8 NK cells due to *Ly49h* structural abnormality. (**A**) Absence of Ly-49H expression on BXD-8 NK cells, as indicated by flow cytometry. Red blood cell lysed splenocytes from C57BL/6, BXD-8, and DBA/2 mice were stained for expression of the indicated Ly-49 receptors (*15*). Cells displayed in the contour plot had been gated to exclude CD3$^+$ and propidium iodide$^+$ (dead) cells. mAb DX5 was used to stain NK cells in DBA/2 mice, because the NK1.1 antigen is not expressed in this strain. The numbers in the upper right quadrant of each contour plot represents the percentage of NK cells (NK1.1$^+$ or DX5$^+$) expressing the indicated Ly-49 receptor. (**B**) Absence of Ly-49H function in BXD-8 NK cells. Human Daudi B lymphoma target cells were incubated with interleukin-2 (IL-2)–activated NK cells generated from C57BL/6 (squares), BXD-8 (circles), or DBA/2 (triangles) mice. Left panel: mAb 3D10 (anti–Ly-49H, solid symbols) induces lysis of Daudi target cell lines by C57BL/6 IL-2–activated NK cells but not by BXD-8 or DBA/2 IL-2–activated NK cells (*15*). mAb B8-24-3 isotype control (anti-K^b, open symbols) does not induce lysis of target cells, demonstrating the specificity of activation by mAb 3D10. Right panel: mAb 4E4 (anti–Ly-49D, solid symbols) induces lysis of Daudi targets by both C57BL/6 and BXD-8 IL-2–activated NK cells but not by DBA/2 effector cells (*29*). Isotype control mAb AF6-88.53 (anti-K^b , open symbols) fails to induce target cell lysis. Similar natural killing ability of NK cells was shown by all three strains against YAC-1 targets (*8*). (**C**) Southern analysis of C57BL/6, DBA/2, and BXD-8 genomic DNA reveals a structural lesion in the BXD-8 *Ly49h* gene. Approximately 10 μg of liver DNA from each strain was digested with either Bgl II or Eco RI, fractionated in 1% agarose, and transferred to Hybond-N. Ly-49G1 (full-length) and Ly-49H (177–base pair Bgl II–Pst I) cDNA probes were labeled with [^{32}P]dCTP using the Rediprime II random primer labeling kit (Amersham) and hybridized overnight at 42°C to duplicate blots as indicated. Final washing was done in 0.2× saline sodium phosphate EDTA and 0.1% SDS at 55°C (Ly-49G1) or 50°C (Ly-49H) for 20 min. Molecular weight markers are noted in kilobases on the left. C57BL/6 and BXD-8 are identical at the *Ly49g* locus and other *Ly49* loci (*8*), but differ at *Ly49h*. Note the absence of *Ly49h*-specific bands at 3.5 kb (Bgl II digest) and 4.9 kb (Eco RI digest) in BXD-8 relative to C57BL/6 (arrows).

Northern and reverse transcription polymerase chain reaction analyses demonstrated that Ly-49H transcripts were absent (*8*), which is consistent with a structural abnormality in the *Ly49h* gene that was demonstrable by Southern analysis (Fig. 2C). By contrast, the expression and genes of other Ly-49 molecules were unaltered and corresponded to the NKC^{B6} haplotype in BXD-8 (Fig. 2, A and C) (*8*). The selective absence of Ly-49H in a susceptible mouse, despite an otherwise intact NKC^{B6} (resistant) haplotype, provides strong genetic evidence in support of the involvement of the Ly-49H NK cell receptor in the control of MCMV infection.

To obtain substantiating immunological data in wild-type C57BL/6 mice, we injected the 3D10 anti–Ly-49H before MCMV inoculation. This treatment markedly elevated viral titers to a level comparable to that shown with anti-NK1.1 (Fig. 3A), which effectively eliminates all NK cells (*16*), and to the MCMV-susceptible phenotype in BALB/c mice. There was no effect of anti–Ly-49H in the liver (*8*), analogous to the *Cmv1*^{r} phenotype, or in spleens from mice treated with control antibodies (Fig. 3A) (*16–18*). Although the paucity of available mAbs against these receptors limited strict control of antibody isotypes, intact anti–Ly-49H treatment did not alter NK cell number or subset distribution (*16*). Furthermore, $F(ab')_2$ anti–Ly-49H administration also abrogated resistance, whereas $F(ab')_2$ control fragments had no effect (Fig. 3B), suggesting that anti–Ly-49H may mediate its effect by blocking Ly-49H recognition of its putative cognate ligand. Anti–Ly-49H also dramatically altered the survival of C57BL/6 mice infected with MCMV, whereas control antibodies had minimal or no effect (Fig. 3C). Most mice died within 1 week of infection.

Taken together, these data unequivocally indicate that the C57BL/6-derived Ly-49H NK cell activation receptor is specifically involved in resistance to MCMV infection in vivo and accounts for the *Cmv1*^{r} phenotype. Our previous genetic data tentatively identified a region telomeric to *Ly49h* in MCMV resistance (*11*, *19*), and it remains possible that this region may be a modifier of Ly-49H expression or function. Regardless, the identification of Ly-49H as a resistance factor for MCMV infection suggests that it may be involved in defense against other pathogens for which resistance loci have been genetically mapped to the NKC (*13*). Alternatively, other related receptors may be specific for these pathogens.

Presumably, the ligand for Ly-49H is expressed on infected cells and may be related to MHC class I molecules, because these are the known ligands for other Ly-49 receptors (*20*). However, MCMV, like other herpesviruses, has evolved numerous strategies to down-regulate MHC class I expression and evade cytotoxic T cells (*6*). Although MCMV encodes an MHC class I–like molecule, it would be counterproductive (from the virus point of view) as a ligand for Ly-49H, and it may instead engage inhibitory Ly-49 receptors (*21*). Alternatively, MCMV infection could induce a host ligand for Ly-49H that is not present normally, similar to the proposed induced expression of ligands for the NKG2D activation receptor in pathologic circumstances (*22*). Conversely, it is possible that the ligand is constitutively expressed but does not trigger Ly-49H until NK cells are released from the inhibitory influence of MHC class I due to down-regulation by MCMV. This latter possibility resonates with predictions deduced from the "missing-self" hypothesis (*23*).

Unlike T and B cells, individual NK cells do not express clonally restricted, rearranged activation receptors. Rather they express a multitude of receptors with overlapping subpopulations expressing the same receptors (*15*). This preformed, widely expressed receptor repertoire presumably confers on NK cells the capacity for early innate control of pathogens during the prolonged period required for clonal expansion of antigen-specific T and B cells. One hallmark of innate immunity, however, has been the involvement of "pattern recognition receptors" that use signaling pathways distinct from the Syk family tyrosine kinase cascade stimulated by ITAM-coupled T and B cell antigen receptors (*24*). Although the range of pathogen specificity of Ly-49H needs to be examined further, it more closely resembles the T cell receptor and B cell receptor because of ITAM association and coupling to the Syk tyrosine kinase pathway. Thus, despite their distinct properties, NK cells resemble T and B cells in innate host responses to pathogens in a manner not previously appreciated.

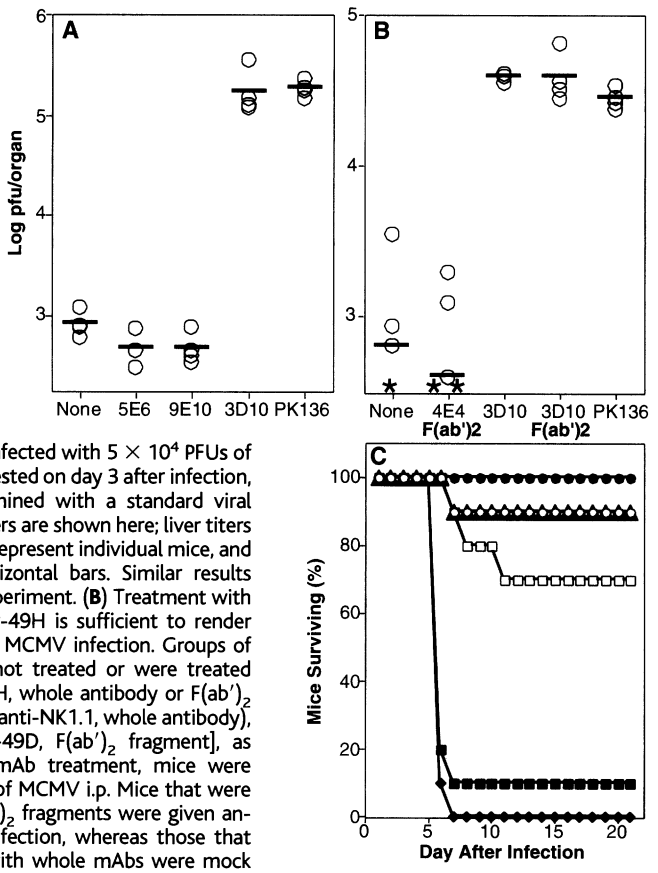

Fig 3. Treatment with anti–Ly-49H results in MCMV susceptibility in otherwise resistant C57BL/6 mice. (**A**) Treatment with mAb 3D10 renders C57BL/6 mice susceptible to MCMV infection. Groups of C57BL/6 mice were either untreated or treated with an intraperitoneal (i.p.) injection of 200 μg of affinity-purified mAb 5E6 (IgG_{2a} anti–Ly-49C/I), 9E10 (IgG_1 isotype control), 3D10 (IgG_1 anti–Ly-49H), or PK136 (IgG_{2a} anti-NK1.1) as indicated (*16*). Two days after mAb treatment, mice were infected with 5×10^4 PFUs of MCMV i.p. Organs were harvested on day 3 after infection, and viral titers were determined with a standard viral plaque assay. Only spleen titers are shown here; liver titers are shown online (*8*). Circles represent individual mice, and means are depicted by horizontal bars. Similar results were obtained in a repeat experiment. (**B**) Treatment with $F(ab')_2$ fragments of anti–Ly-49H is sufficient to render C57BL/6 mice susceptible to MCMV infection. Groups of C57BL/6 mice were either not treated or were treated with mAb 3D10 [anti–Ly-49H, whole antibody or $F(ab')_2$ fragment], with mAb PK136 (anti-NK1.1, whole antibody), or with mAb 4E4 [anti–Ly-49D, $F(ab')_2$ fragment], as indicated. Two days after mAb treatment, mice were infected with 5×10^4 PFUs of MCMV i.p. Mice that were previously treated with $F(ab')_2$ fragments were given another dose on the day of infection, whereas those that were untreated or treated with whole mAbs were mock treated. Splenic viral titers were determined on day 3 after infection. There were four mice in each group, except for the mAb 4E4–treated group, which had five. Asterisks indicate individual mice in each group that had titers below the level of detection of the plaque assay. Means are depicted as horizontal bars. To determine the mean, the minimum number of detectable PFUs (10^2) was assumed for mice with titers below the level of detection of the assay. This assumption overestimates the mean for those groups having titers below detectable levels. (**C**) C57BL/6 mice treated with mAb 3D10 succumb to a sublethal dose of MCMV. Groups of C57BL/6 mice (10 per group) were either not treated (open circles) or were treated with mAb 5E6 (open squares) (IgG_{2a} anti–Ly-49C/I), mAb 9E10 (solid circles) (IgG_1 isotype control), mAb 4E4 (triangles) (IgG_{2a} anti–Ly-49D), mAb 3D10 (solid squares) (IgG_1 anti–Ly-49H), or mAb PK136 (diamonds) (IgG_{2a} anti-NK1.1). Two days after mAb treatment, mice were infected with 10^5 PFUs of MCMV i.p. and were followed for 21 days after infection. The percentage of mice surviving within each group was determined daily.

References and Notes

1. A. B. Bakker, J. Wu, J. H. Phillips, L. L. Lanier, *Hum. Immunol.* **61**, 18 (2000).
2. C. A. Biron, K. B. Nguyen, G. C. Pien, L. P. Cousens, T. P. Salazar-Mather, *Annu. Rev. Immunol.* **17**, 189 (1999).
3. C. A. Biron, K. S. Byron, J. L. Sullivan, *N. Engl. J. Med.* **320**, 1731 (1989).
4. J. F. Bukowski, B. A. Woda, S. Habu, K. Okumura, R. M. Welsh, *J. Immunol.* **131**, 1531 (1983).
5. C. H. Tay, R. M. Welsh, *J. Virol.* **71**, 267 (1997).
6. D. Tortorella, B. E. Gewurz, M. H. Furman, D. J. Schust, H. L. Ploegh, *Annu. Rev. Immunol.* **18**, 861 (2000).
7. A. A. Scalzo, N. A. Fitzgerald, A. Simmons, A. B. La Vista, G. R. Shellam, *J. Exp. Med.* **171**, 1469 (1990).
8. Supplemental text and figures are available on *Science* Online at www.sciencemag.org/cgi/content/full/292/5518/934/DC1
9. A. A. Scalzo *et al.*, *J. Immunol.* **149**, 581 (1992).
10. A. A. Scalzo *et al.*, *Genomics* **27**, 435 (1995).
11. C. A. Forbes *et al.*, *Genomics* **41**, 406 (1997).
12. C. Depatie, E. Muise, P. Lepage, P. Gros, S. M. Vidal, *Genomics* **39**, 154 (1997).
13. M. G. Brown, A. A. Scalzo, K. Matsumoto, W. M. Yokoyama, *Immunol. Rev.* **155**, 53 (1997).
14. For strain distribution profile and genotyping data, see www.informatics.jax.org/searches/riset_form.shtml.
15. H. R. Smith *et al.*, *J. Exp. Med.* **191**, 1341 (2000).
16. Anti-NK1.1 depletion was verified by the absence of staining with the DX5 mAb in antibody-treated mice. Intact anti–Ly-49H treatment did not alter the percentage of NK1.1$^+$ or Ly-49D$^+$ (coexpressed on most Ly-49H$^+$ cells) CD3$^-$ spleen cells as analyzed by flow cytometry. Although mAb 5E6 (anti–Ly-49C/I) reacts with a similar percentage of NK cells as does anti–Ly-49H, it had no effect, in agreement with previous observations indicating that administration of antibodies against Ly-49A, C/I, D, or G2 did not alter MCMV resistance (*18*, *19*).
17. C. H. Tay *et al.*, *J. Immunol.* **162**, 718 (1999).
18. C. Depatie *et al.*, *Int. Immunol.* **11**, 1541 (1999).
19. M. G. Brown *et al.*, *J. Immunol.* **163**, 1991 (1999).
20. F. M. Karlhofer, R. K. Ribaudo, W. M. Yokoyama, *Nature* **358**, 66 (1992).
21. H. E. Farrell *et al.*, *Nature* **386**, 510 (1997).
22. W. M. Yokoyama, *Nature Immunol.* **1**, 95 (2000).
23. K. Karre, in *Mechanisms of Cytotoxicity by NK Cells*, R. B. Herberman, D. M. Callewaert, Eds. (Academic Press, Orlando, FL, 1985), pp. 81–92.
24. A. Aderem, R. J. Ulevitch, *Nature* **406**, 782 (2000).
25. J. A. Blake, J. T. Eppig, J. E. Richardson, M. T. Davisson, Mouse Genome Informatics Web site (www.informatics.jax.org) (2000).
26. M. G. Brown *et al.*, *Genomics* **42**, 16 (1997).
27. C. Depatie *et al.*, *Genomics* **66**, 161 (2000).
28. K. L. McQueen, S. Lohwasser, F. Takei, D. L. Mager, *Immunogenetics* **49**, 685 (1999).
29. A. H. Idris *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6330 (1999).

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